Kaempferol treatment ameliorates memory impairments in STZ-induced neurodegeneration by acting on reelin signaling

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INTRODUCTION

According to the World Health Organization’s report, Alzheimer’s disease (AD), which constitutes 60–70% of all dementia cases, is a neurodegenerative disease that negatively affects memory, speech, and problem-solving in daily life. In addition, the majority of AD patients are individuals over the age of 65 with late-onset (sporadic) AD (LOAD) (Heneka et al., 2015). Senile plaques, neurofibrillary tangles, and neuroinflammation are suggested causes of AD pathology (Heneka et al., 2015). Much of the current research has shown that oxidative stress is closely associated with many neurodegenerative diseases, in-
cluding AD (Abromov et al., 2004). In AD pathology, oxidative stress plays a major role in the formation and progression of neuroinflammation by microglial activation (Heneka et al., 2015; Kinney et al., 2018). Today, these situations direct drug design toward targets that prevent or treat neuroinflammation by altering the negative impact of microglial activation (Hampel et al., 2020). In addition, the fact that the drugs used today only alleviate the symptoms of the disease but cannot stop its progression has prompted researchers to find new traditional medications based on neuroprotective and anti-inflammatory properties (Dalli et al., 2018). Antioxidant activities of plant-derived flavonoids – Gingko biloba, kaempferol (KMP), and quercetin, have been demonstrated in in vivo and in vitro experiments (Calderon-Montano et al., 2011).

KMP (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one) is a secondary metabolite derived from fruits and vegetables. Besides being used for years in alternative medicine, with beneficial effects on chronic and acute inflammatory diseases as well as cancer, current studies have expanded the usage of KMP to vascular endothelial inflammation and hypertrophic scarring (Ren et al., 2019). The most well-known function of KMP is as an antioxidant flavonoid with four hydroxy groups that reduce oxidative stress (Ren et al., 2019). The antioxidant capacity of KMP makes it a good candidate for treating neurodegenerative diseases. According to the molecular and microscopic studies done by Filomeni et al. (2012), examining various polyphenol molecules, KMP was effective against both oxidative stress and apoptosis in human neuroblastoma SH-SY5Y cells in an acute toxicity model of Parkinson’s disease induced by rotenone. In AD pathology, it was noted that KMP exerts a neuroprotective effect by inhibiting acetylcholinesterase activity to improve cognitive functions (Zarei et al., 2019). This flavonoid demonstrates antioxidant actions by positively modulating the antioxidant enzymes such as glutathione and superoxide dismutase (SOD) to prevent the formation and progression of AD pathology (Kouhestani et al., 2018). In addition to these studies, the neuroprotective actions of KMP occur by decreasing amyloid beta accumulation and increasing BDNF and CREB expression, which are important for synaptic plasticity and for the inhibition of the activation of inflammatory pathways (Park et al., 2011; Hanaki et al., 2016; Yan et al., 2019). However, there are few studies related to the effects of KMP on memory improvement in neurodegenerative diseases through its action on memory-related proteins. Therefore, in the present study, we aimed to investigate the underlying molecular mechanisms of cognitive recovery after neurodegeneration resulting from KMP treatment by measuring changes in the expression of memory-related molecules in rats.

**Experimental design**

**Animals**

Adult male Long-Evans rats (12-week-olds, weighing 300–350 g) were supplied from the animal research and breeding center of Bezmialem Vakif University to investigate the protective effects of KMP against neurodegeneration. The experimental procedures were carried out according to the NIH guidelines (NIH publication No. 85–23, revised 1996) regarding the care and use of the experimental animals’ standards (12 h light/dark cycles, 22°C, and 60% humidity) with *ad libitum* food and water. All experimental procedures were approved by the Committee for Animal Research Ethics at Bezmialem Vakif University (2019/267) and were designed to minimize suffering in accordance with ARRIVE guidelines.

**Intracerebroventricular (i.c.v) injection of streptozotocin (STZ)**

The STZ-induced neurodegeneration model was adapted from previous studies (Isik et al., 2009; Dalli et al., 2018). STZ (Sigma, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (aCSF) and given to the rats by i.c.v injection bilaterally (0.8 mm anteroposterior, 1.5 mm mediolateral, and 3.5 mm dorsoventral) at a dose of 3 mg/kg per animal. In addition, aCSF was given to the rats by i.c.v injection (20 µl) to obtain the sham control. STZ and aCSF injections were applied twice 48 h apart for each rat, and 28 days after the second STZ injection, KMP treatment was started. The timeline of the experimental study is presented in Fig. 1.

At the beginning of the experiments, rats were randomly divided into three experimental groups: the Sham control group, receiving i.c.v aCSF injection (n=5); the STZ group, receiving i.c.v. STZ injection (n=6); and the STZ+KMP group, receiving i.c.v. STZ injection plus KMP treatment (n=7). After the second STZ injection, the rats were left in their home cage for 28 days to develop STZ-induced neurodegeneration. Then, the STZ+KMP group was subjected to KMP (10 mg/kg, i.p.) treatment for 12 days (Lagoa et al., 2009; Yang et al., 2019). To make the KMP solution, 100 mg KMP (K0133-50MG, Sigma-Aldrich) was dissolved with 400 µl DMSO in 10 ml saline. In addition, STZ and Sham control groups received i.p. injections of the KMP solvent for 12 days (Fig. 1).
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Morris water maze (MWM)

At the end of the KMP treatment, the MWM test was performed. The MWM tank (150 cm in diameter and 60 cm in height) was filled with water up to a height of 45 cm. Milk powder was used to make the water opaque, and cues were placed around the room for place learning. Rats were trained in the MWM after the KMP treatment, as described previously (Dalli et al., 2018). Over five consecutive days, rats performed a daily session of four trials in the MWM with a hidden platform (11 cm × 11 cm) for place learning. For each trial, the rat was allowed to swim for a maximum of 60 s. If they found the hidden platform using extra-maze cues, the rat was taken from the platform and returned to the home cage. Inter-trial intervals were five minutes, and each trial was recorded by a video-tracking system (EthoVision XT11 software, Noldus Information Technology, Wageningen, Netherlands) for swim trajectory, swim velocity, escape latency, and swimming distance. A probe trial was performed the day after place learning to evaluate memory performance. The platform in the MWM tank was removed, and the rats were put into the water for 60 s. In the probe trial, the percentage of time that the rat spent in the platform quarter and in the imaginary 40 cm diameter annulus (A40) around the escape platform location were calculated for each rat (Dalli et al., 2018).

Molecular analysis

After the behavioral experiments were completed, the anesthetized animals were sacrificed by decapitation. The hippocampi were dissected and frozen on dry ice to store at -80°C until molecular analysis.

For protein analysis, the hippocampal tissue was homogenized with RIPA lysis buffer in a bead homogenizer. The protein concentrations were determined with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham) using a MultiskanTM GO Microplate Spectrophotometer. Equal amounts of proteins from each rat were used for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% Tris-Glycine gels (Mini-Protean, BIORAD). Afterward, proteins were transferred to the PVDF membranes and the membranes were blocked with 5% milk powder in 0.1 % Tween 20/0.1 M Tris-buffered saline (TBST). They were incubated overnight at 4°C with primary antibodies against glutamate decarboxylase 67 (GAD67, mouse, Chemicon, Germany), reelin (mouse, Chemicon, Germany), prealbumin (sheep, Abcam, USA), klotho (rabbit, Abcam, USA), phosphorylated CAMKII (rabbit, Cell Signaling Technology, USA), phosphorylated NMDAR (rabbit, Cell Signaling Technology, USA) and β-tubulin (protein loading control, rabbit Abclonal, U.K). Each primary antibody was diluted in 5% milk powder in 0.1% Tween 20/0.1 M TBST in a 1:1000 dilution. The next day, the membranes were washed with TBST and incubated with secondary antibodies (1:5000: anti-rabbit – Cell Signaling Technology, USA; anti-mouse – Cell Signaling Technology, USA; and anti-sheep – Invitrogen, USA) in 5% milk powder in 0.1% Tween 20/0.1 M TBST for an hour at room temperature. Afterward, the PVDF membrane was visualized with a chemiluminescent substrate under a CCD camera in the Fusion FX7 (Vilber Lourmat) system. ImageJ analysis system (National Institute of Mental Health, Bethesda, MD, USA) was used for densitometric quantification of immunoreactivity.

Statistical analysis

The mean values and standard deviations were calculated for behavioral and molecular data. Two-way repeated measure ANOVA with Fisher’s Least Significant Difference (LSD) test for pairwise comparisons was applied to the MWM data using treatment and day as independent factors. A one-way ANOVA test was performed to calculate the differences between groups for probe trial and molecular data. The SPSS 18 statistical package was used for statistical analysis. The statistical significance value was accepted as $P \leq 0.05$. $P$ values between 0.10 and 0.05 were accepted as marginally significant.
RESULTS

In the current study, according to two-way repeated measure ANOVA (treatment X day), there were no between-group differences ($F_{(2,17)}=0.203$, $P=0.818$ and $F_{(2,17)}=0.079$, $P=0.924$) or day × group interactions ($F_{(8,60)}=0.792$, $P=0.612$ and $F_{(8,60)}=0.896$, $P=0.526$) for latency or distance swam to the escape platform (Fig. 2A, 2B). In addition, there was no difference in the swim velocity among the groups ($p>0.05$) (data not shown). However, as seen from the swimming trajectory, a significant decrease day by day in the latency and the distance swam to reach the hidden platform ($F_{(4,60)}=48.706$, $P<0.001$ and $F_{(4,60)}=77.530$, $P<0.001$) was observed in MWM training (Fig. 2A, 2B, and 2C). In accordance with the results of the post hoc LSD test, an increase in the latency to reach the hidden platform on the fifth day of training ($P=0.05$) and an increase in the distance to reach the hidden platform on the fourth day of training ($P=0.05$) was observed in the STZ group compared to the sham control group. During MWM training, the STZ+KMP group showed similar learning performances to the sham control group.

For the MWM probe trial, which shows the time spent in the platform quadrant without an escape platform (Fig. 3A), there was a small difference between the groups in the percentage of time spent in the platform quadrant ($F_{(2,17)}=3.354$, $P=0.073$) (Fig. 3B), but there was no significant change between the groups in the time spent in the A40 ($F_{(2,17)}=1.655$, $P=0.229$) (Fig. 3C). As seen in Fig. 3B, a significant decrease in the percentage of time spent in the platform quadrant was noted in the STZ group compared to the sham control group ($P=0.035$). On the other hand, KMP treatment increased the time spent in the platform quadrant compared to the STZ group ($P=0.063$), approaching the control values ($P=0.445$) (Fig. 3B).

Effects of KMP on the hippocampal molecular composition were evaluated by analyzing several memory proteins after STZ-induced neurodegeneration. In the present study, there was a marginally significant difference in the phosphorylation of NMDAR ($F_{(2,13)}=3.406$, $P=0.074$). KMP treatment significantly increased the level of phosphorylated NMDAR, which was decreased with STZ induction ($P=0.054$ and $P=0.041$, respectively) (Fig. 4).

![Fig. 2. Behavioral tasks carried out to evaluate the functional effects of KMP treatment on learning and memory in the rats. (A) Escape latency; (B) the distance to reach the hidden platform, which is a cue for learning skills in the MWM for the Sham control (n=5), STZ (n=6), and STZ+KMP (n=7) groups; and (C) the swimming trajectory on Day 1 and Day 5 for a rat in the Sham control group. Data are presented as mean ± standard deviation, and * indicates significance relative to the control for $P<0.05$.](image-url)
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However, both STZ induction and KMP treatment did not produce any change in the phosphorylation of CAMKII, an important mediator of learning and memory (Fig. 5).

Furthermore, the expression level of reelin protein, a large extracellular matrix glycoprotein involved in learning and memory, was significantly altered by treatment. In our study, we observed that the expression of both the 200 kDa and 300 kDa subunits of reelin decreased in the hippocampus of rats with STZ-induced neurodegeneration in parallel to the decrease in the phosphorylation of NMDAR ($F_{(2,12)}=8.108$, $P=0.008$ and $F_{(2,12)}=4.371$, $P=0.043$, respectively). In addition, KMP treatment restored the level of the reelin protein that increased NMDAR phosphorylation and memory performance (Fig. 6). However, the KMP-related increase was only significant for the 200 kDa reelin subunit compared to the STZ group ($P=0.003$).

In parallel to reelin expression, the level of the GABAergic protein GAD67 slightly decreased in the STZ-induced neurodegeneration model ($F_{(2,12)}=3.679$, $P=0.063$). However, KMP treatment increased the expression of GAD67, approaching the level of the controls ($P=0.027$), supporting its memory enhancement effect through the reelin signaling pathway (Fig. 7).

Fig. 3. (A) The swim trajectory in probe trial 5 for a rat in the Sham control group, (B) the percentage of time spent in the platform quadrant, and (C) the time spent in the Annulus40 by animals during the attempts to find the platform as a measure of memory for the Sham control (n=5), STZ (n=6), and STZ + KMP (n=7) groups. Data are presented as mean ± standard deviation, and * indicates significance relative to the control for $P \leq 0.05$.

Fig. 4. Semiquantitative analysis of phosphorylated NMDA receptor to β-tubulin in the hippocampus by western blotting for the Sham control (n=4), STZ (n=4), and STZ + KMP (n=5) groups. All of the blots were run in duplicate and screened animal by animal. Representative photos belong to the samples pooled for each group. Data are presented as mean ± standard deviation, and * indicates significance relative to the control for $P \leq 0.05$.

Fig. 5. Semiquantitative analysis of phosphorylated CAMKII to β-tubulin in the hippocampus by western blotting for the Sham control (n=4), STZ (n=4), and STZ + KMP (n=5) groups. All of the blots were run in duplicate and screened animal by animal. Representative photos belong to the samples pooled for each group. Data are presented as mean ± standard deviation, and * indicates significance relative to the control for $P \leq 0.05$. 

In opposition to the GAD67 protein level, the klotho protein level, which appears to be involved in aging, significantly increased in the STZ-induction group \( (F_{(2,12)}=25.360, P<0.001, \text{Fig. 8}) \). In addition, KMP treatment significantly increased klotho levels \( (P<0.001) \).

Lastly, the protein expression of transthyretin (pre-albumin) was slightly increased due to STZ induction compared to the sham control group. This increase in the prealbumin expression due to KMP treatment reached significance compared to the control group \( (P=0.048) \) (Fig. 9).
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The primary aim of new treatment strategies for neurodegenerative diseases, like AD, is attenuating cognitive decline and strengthening memory functions to increase the life quality of patients. Therefore, in addition to research related to identifying candidate molecules that can eliminate the factors that cause neurodegeneration, phytochemicals that can increase synaptic plasticity in patients have become an important focus in investigations of treatment strategies (Weller and Budson, 2018). In the present study, we tried to determine whether KMP has an effect on memory-related molecules in ameliorating STZ-induced dysfunctions of learning and memory.

Previous studies demonstrated an ameliorative role for KMP in learning and memory in distinct models of neurodegeneration (Lei et al., 2012; Kouhestani et al., 2018; Das et al., 2018; Babaei et al., 2021). In the present study, the results showed that both in the training sessions and in the probe trial, KMP treatment increased the learning and memory performance that had been impaired by STZ administration, which is consistent with previous studies. Memory enhancement by KMP administration has been explained by its effects on oxidative stress and inflammation, which might be primary causes of neurodegeneration (Kouhestani et al., 2018; Babaei et al., 2021).

To further investigate how these antioxidative and anti-inflammatory effects of KMP act on the molecular mechanisms of learning and memory, we measured the expression of several memory-related molecules after STZ induction and KMP treatment. First, the changes in the expression levels of phosphorylated NMDAR and phosphorylated CAMKII were investigated because of their critical roles in synaptic plasticity. As is well-known, the molecular mechanisms that underly learning and memory formation are related to activity-dependent changes at synapses, referred to as synaptic plasticity, such as long-term potentiation (LTP) (Lynch, 2004). NMDARs play an important role in the regulation of synaptic plasticity and the induction of LTP, and their expression decreases with increasing age (Mota et al., 2014; Burnashev and Szepetowski, 2015; He et al., 2021). Therefore, it was suggested that memory impairment in AD may occur due to synaptic loss due to the downregulation of the expression and phosphorylation of NMDAR rather than Aβ deposition or neurofibrillary tangles (Terry et al., 1991; He et al., 2021). In the present study, decreased NMDAR expression due to STZ induction was ameliorated by KMP treatment. Our results are consistent with previous studies demonstrating that KMP treatment could improve memory function in neurodegeneration by increasing the activity of NMDARs (Park et al., 2014). In another study, where ginkgo flavanols, quercetin, and KMP treatment in AD transgenic mice activated NMDA receptors, it was suggested that the effect of the flavonoids in ameliorating symptoms of the neurodegeneration depended on NMDA receptors to enhance BDNF and CREB signaling (Hou et al., 2010). A neuroprotective KMP effect on learning and memory was also noted during hypoxic stress through the correlation of NMDARs and Trkβ (Das et al., 2018).

The regulation of NMDARs is mainly dependent on protein kinase C (PKC) due to its blocking effect on Mg²⁺ in NMDAR channels to increase intercellular calcium (Li et al., 2011; Horak et al., 2014). In this process of synaptic plasticity, PKC first phosphorylates CaMKII, then promotes the binding of CaMKII and NMDAR to the membrane for memory formation. In the literature, several other studies also showed a strong relation between AD symptoms and CAMKII expression and phosphorylation (Wang et al., 2005; Amada et al., 2005). However, in our study, CAMKII activation did not change due to STZ induction or KMP treatment. Hou et al. (2010) also reported an increase in the NMDAR activity independent of CAMKII activity. Several other studies have also suggested that CaMKII protein expression or its phosphorylation is not altered in the hippocampus of patients in the severe stages of AD (Amada et al., 2005; Reese et al., 2011). In these studies, the changes in CAMKII activity were...
observed in the subcellular distribution, from synapse to cytosol (Gu et al., 2009). While the protective effect of KMP against cardiac sinus node dysfunction was noted in a previous study (An and Kim, 2015), we did not observe any treatment effect of KMP on the activity of CAMKII in the hippocampus of rats with STZ-induced neurodegeneration, as mentioned previously.

Reelin is another synaptic plasticity protein that is affected in neurodegeneration (Yu et al., 2016). In previous studies, a decrease in the level of reelin was noted in both the early phase of AD and before the onset of amyloid pathology (Chin et al., 2007; Herring et al., 2012). During aging, reelin protein aggregates, which reduces its level in the hippocampus; therefore, this reduction in the reelin signaling mechanism can cause AD pathology by increasing Aβ deposition and tau hyperphosphorylation (Kocherhans et al., 2010). A relationship between AD pathology and reelin signaling was indicated due to identical receptors such as apolipoprotein receptor 2 (ApoER2), very-low-density lipoprotein receptor (VLDLR), and Disabled-1 (Dab1) (Yu et al., 2016). In addition, the disruption of reelin signaling could be a potential mechanism underlying memory impairment in neurodegeneration because reelin-induced tyrosine phosphorylation of NMDARs is a key step in the production of LTP (Chen et al., 2005). Therefore, it should be noted that reelin has a modulatory role in learning and memory because of its modulatory role in NMDAR function (Knuesel, 2010). The full-length reelin protein is approximately 400 kDa as a monomer. Under physiological conditions, there are different cleavage sites for reelin which produce fragments with different functions (Lussier et al., 2016). While a 300 kDa subunit is the result of C-t cleavage, a 200 kDa subunit of reelin occurs due to N-t cleavage (Sato et al., 2015). In our study, the KMP-related increase was only significant for the 200 kDa subunit of reelin, which suggests that KMP treatment increased reelin activity by N-t cleavage rather than C-t cleavage. Because investigations have attempted to use reelin as a potential treatment strategy for neurodegeneration (Rogers et al., 2011; Ishii et al., 2015), molecules such as KMP, which increase reelin expression, can be candidate therapeutic targets for AD pathology.

Previous studies have shown that reelin is secreted by GABAergic neurons that regulate NMDAR homeostasis in the postnatal hippocampus (Campo et al., 2009). Therefore, to obtain more detail regarding the molecular mechanism of KMP in memory improvement in neurodegeneration, we measured the level of GAD67 protein, which is a GABAergic neuron marker. The downregulation of GAD67 protein in neurodegeneration and an ameliorative effect of flavonoids on GAD67 levels were also noted in previous studies (Seidl et al., 2001; Govindpani et al., 2020; Ben-Azu et al., 2020). Ben-Azu and his colleagues (2020) found a relationship between the upregulation of GAD67 and the attenuation of oxidative stress and neuroinflammation due to flavonoid consumption in preventing cognitive impairment. Therefore, the memory-enhancing effect of KMP may be related to its antioxidative and anti-inflammatory action on reelin signaling.

The level of klotho, known as an aging protein, increased in the STZ-induction group, opposite to the level of GAD67. This was consistent with previous studies (Reish et al., 2013) and can be explained as a compensatory mechanism for a decrease in GABAergic functions that results in learning and memory impairment. In a mouse study, the researchers observed that an increase in levels of klotho enhanced LTP and eliminated the cognitive impairments (Dubal et al., 2014). In addition, KMP treatment increased the level of klotho, as other flavonoids have been shown to do (Li et al., 2019).

Similar to klotho levels, prealbumin levels were also slightly increased in the STZ-induced neurodegeneration group in our study, while the level of prealbumin generally decreases in the early phase of AD (Gião et al., 2021). It should be mentioned that prealbumin provides neuroprotection against AD because it removes Aβ (Silva et al., 2017). This may also be a compensatory mechanism against neurodegeneration in the brain. On the other hand, the increase in the prealbumin expression can be explained by its relationship with glucose metabolism. In a previous study, it was observed that levels of prealbumin increased in type 2 diabetes, which partially reflects our disease model (Mody et al., 2008). A small number of studies showed the presence of prealbumin in the hippocampus, cortex, and amygdala (Puskás et al., 2003; Stein and Johnson, 2002). It was suggested that the body tries to normalize prealbumin levels to prevent disease conditions by activating other brain regions (Alemi et al., 2021). In addition to the presence of prealbumin in the serum and CSF, the expression of prealbumin has been described to occur in the choroid plexus and the meninges of the brain (Sousa et al., 2007). Therefore, another possibility for the increase in the prealbumin level may be related to blood or CSF contamination because our animals were not perfused. According to previous studies, by having more hydroxyl groups, flavonoids like KMP decrease the degree of conversion to amyloid fibrils by stabilizing the prealbumin tetramer due to interactions with the T4 binding sites (Baures et al., 1998; Radović et al., 2006).
CONCLUSIONS

In summary, we observed that KMP treatment has a role in ameliorating the observed memory impairment by acting on the GAD67/reelin/NMDAR signaling pathway in neurodegeneration. According to the overall results of the present study, KMP may be a candidate molecule for the elimination of AD symptoms due to its capacity to recover neurodegeneration-related learning and memory impairments by increasing synaptic plasticity.

REFERENCES


